Genetics of Adeno-Associated Virus: Isolation and Preliminary Characterization of Adeno-Associated Virus Type 2 Mutants

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We constructed insertion and deletion mutants with mutations within the adeno-associated virus (AAV) sequences of the infectious recombinant plasmid pSM620. Studies of these mutants revealed at least three AAV phenotypes. Mutants with mutations between 11 and 42 map units were partially or completely defective for rescue and replication of the AAV sequences from the recombinant plasmids (*rep* mutants). The mutants could be complemented by mutants with replication-positive phenotypes. The protein(s) that is affected in *rep* mutants has not been identified, but the existence of the *rep* mutants proves that at least one AAV-coded protein is required for viral DNA replication. Also, the fact that one of the *rep* mutant mutations maps within the AAV intron suggests that the intron sequences code for part of a functional AAV protein. Mutants with mutations between 63 and 91 map units synthesized normal amounts of AAV duplex DNA but could not generate single-stranded virion DNA (*cap* mutants). The *cap* phenotype could be complemented by *rep* mutants and is probably due to a defect in the major AAV capsid protein, VP3. This suggests that a preformed capsid or precursor is required for the accumulation of single-stranded AAV progeny DNA. Mutants with mutations between 48 and 55 map units synthesized normal amounts of AAV single-stranded and duplex DNA but produced substantially lower yields of infectious virus particles than wild-type AAV (*lip* mutants). The *lip* phenotype is probably due to a defect in the minor capsid protein, VP1, and suggests the existence of an additional (as yet undiscovered) AAV mRNA. Evidence is also presented for recombination between mutant AAV genomes during lytic growth.

Adeno-associated virus (AAV) is a defective parvovirus that requires coinfection with a helper virus for lytic growth (1, 12, 13, 18, 31, 57; see references 3, 4, 13, and 15 for reviews.) Members of both the adenovirus and herpesvirus families are capable of providing helper functions (1, 8, 13, 18, 31, 57), and the host range of AAV appears to be determined in most cases by the host range of the helper virus (7, 13, 18, 31, 44). In the absence of a helper, AAV can integrate into a mammalian chromosome through its terminal repeat sequences and persist as a latent infection (3, 5, 19, 26, 32; A. K.-M. Cheung, Ph.D. dissertation, University of Florida, Gainesville, 1979). Latent AAV genomes do not express their genes but are capable of being rescued when their hosts are superinfected with a helper virus (3, 5, 19, 26, 32).

Because AAV is defective (i.e., it does not plaque by itself), it has been difficult to study the biology of the virus by using conventional genetic techniques. Nevertheless, a large amount of information about the genetics of AAV has been accumulated as the result of studies on the proteins (9, 10, 35–38, 50; R. A. McPherson and J. A. Rose, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, S25, p. 288) and mRNAs (11, 15, 23–25, 43, 45, 66) of the virus and by the determination of the complete nucleotide sequence of the genome (21, 23, 45, 47, 66). AAV has a linear single-stranded (2, 6, 48, 61) genome (4,675 bases long) with an inverted terminal repetition of 145 bases (21, 40, 47, 66). The terminal repetition contains several palindromes that allow the terminal sequence to form a hairpin structure (47) which is thought to be used for the initiation of DNA replication (4, 27, 46, 64, 67). (For a review of the AAV DNA replication mechanism, see reference 4.) In addition to their role in DNA replication, the AAV termini are believed to be involved in AAV integration and rescue (3, 19, 64), although relatively little is known about the mechanism of these events.

Three major RNA transcripts (2.3, 3.3, and 3.9 kilobases [kb]) have been identified (Fig. 1) during AAV lytic infections (15, 23–25, 43, 45, 66). These RNAs share a common intron (41 to 48 map units [m.u.]) and a common polyadenylation site (95 m.u.). In addition, an unspliced polyadenylated version of each RNA (2.6, 3.6, and 4.2 kb) has been reported (15, 25, 43). It is not known whether these unspliced transcripts code for functional proteins.

Computer analysis of the AAV DNA sequence has revealed two major open reading frames in the AAV genome (66). The major open reading frame on the right side of the genome (60 to 93 m.u.) can code for a protein of approximately 63 kilodaltons (504 amino acids) and presumably is expressed from the most abundant spliced mRNA (2.3 kb) synthesized during AAV infections. Comparison of the amino acid composition of the hypothetical protein coded by this region with the amino acid composition and size (60 to 67 kilodaltons) of the major AAV capsid protein, VP3, indicates that they are the same (62, 66). In vitro translation experiments, however, indicate that the 2.3-kb mRNA is capable of synthesizing not only VP3, but VP1 (85 to 90 kilodaltons) and VP2 (72 to 73 kilodaltons) as well (35). Furthermore, peptide mapping indicates that the three capsid proteins have most of their amino acid sequences in common (37). Thus, it appears that the region from 60 to 93 m.u. probably codes for all of the VP3 amino acid sequence and most of VP1 and VP2. However, the positions of the additional coding regions required to synthesize VP1 and VP2 are uncertain.

Relatively little is known about the other major open reading frame in AAV (7 to 48 m.u.) which presumably can be expressed from the spliced 3.9-kb (or unspliced 4.2-kb)

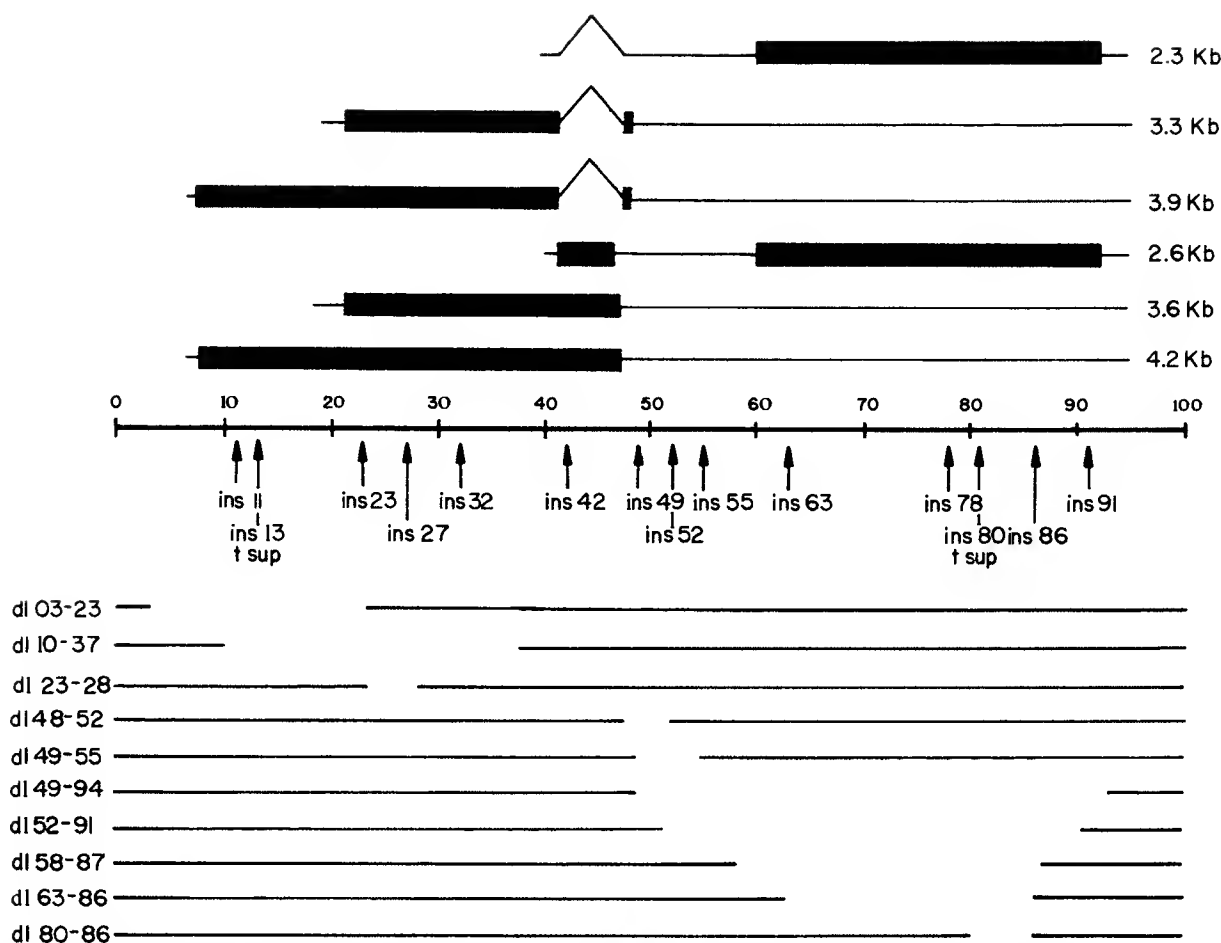---

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FIG. 1. Physical structure and position of AAV RNAs, open reading frames, and mutants. Above the map position line, the solid lines indicate the portions of the genome that are present within the major AAV spliced (2.3, 3.3, and 3.9 kb) and unspliced (2.6, 3.6, and 4.2 kb) RNAs (11, 15, 23–25, 43, 45, 66). The solid boxes indicate the position of the major open reading frame in each transcript that is likely to be translated (45, 66). Below the map position line, the arrows indicate the positions of insertion mutations and the interrupted lines indicate the positions of AAV sequences missing in the deletion mutants.

RNA (Fig. 1). Because a portion of this open reading frame is also present in the spliced 3.3-kb (and the unspliced 3.6-kb) mRNA, it has been suggested that the reading frame from 7 to 48 m.u. codes for at least two AAV noncapsid proteins (45, 66). These hypothetical, nonstructural proteins have not been conclusively identified in AAV-infected cell extracts, nor is their function(s) known.

Finally, several other shorter open reading frames (66) have been identified (Fig. 1). Among these, one is located entirely within the intron (41 to 46 m.u.), and another is at 47 to 54 m.u. Nothing is known about the possible function of these open reading frames.

As already mentioned, it has been difficult to apply conventional genetic techniques to the AAV genome. To circumvent this problem, we have taken advantage of the fact that AAV-pBR322 recombinant plasmids are infectious (63). When such plasmids are transfected into human cells with adenovirus 2 (Ad2) as helper, the AAV genome is rescued and a normal lytic cycle ensues. This offered a unique opportunity for genetic analysis. Because the nucleotide sequence of AAV was known, it was possible to target mutations to any region of AAV within the recombinant plasmid, to clone and amplify the mutant plasmid in *Escherichia coli*, and then to directly assess the mutant phenotype

by transfection into mammalian cells. In a previous report, we used this approach to examine the phenotypes of mutations within the AAV terminal sequences (64). In this report, we describe the phenotypes of mutations within the internal coding regions of AAV.

MATERIALS AND METHODS

Cells. Adenovirus-transformed human cells, 293-31 (22), were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum, 1% glutamine, penicillin, and streptomycin. HeLa and Detroit 6 cells were maintained in minimal essential medium containing 10% fetal calf serum, 1% glutamine, penicillin, and streptomycin.

Enzymes and linkers. Restriction enzymes, T4 ligase, T4 DNA polymerase, T4 polynucleotide kinase, *E. coli* DNA polymerase I, and the Klenow fragment were purchased from New England Biolabs and Bethesda Research Laboratories, and the enzyme reactions were performed according to the suppliers' specifications. *Bgl*II linkers were purchased from New England Biolabs.

DNA transfection and virus infection. Cells were transfected by the DEAE-dextran method (49) as described previously (51), except that 1 to 11 μg of helical closed circular form I plasmid DNA was used per 10-cm dish. After transfection,

the cells were infected with Ad2 virus at a multiplicity of infection of 5.

AAV wild-type and mutant virus stocks were prepared by freezing and thawing infected cells three times at 36 to 48 h posttransfection. The medium was then heated to 56°C for 10 min.

Mutant virus titers were determined by immunofluorescence (14) with anti-AAV capsid antibody (a kind gift of M. D. Hoggan) or by quantitating the amount of AAV DNA synthesized in cells infected with samples of the viral stock. In the latter method, virus stocks from wild-type and mutant plasmid transfections were used to infect human cells, and the yields of monomer-length duplex DNA in mutant and wild-type infections were compared.

DNA extraction and hybridization procedure. Low-molecular-weight DNA was isolated from tissue culture cells at 24 to 48 h after transfection or infection by the method of Hirt (30) as previously described (51), with the exception that the samples were treated with 0.8 mg of pronase per ml which had been predigested for 1 h at 37°C. The DNA was fractionated by electrophoresis on 1.4% agarose gels, transferred to nitrocellulose (65), and annealed to ^{32}P-labeled nick-translated DNA (0.8 × 10^8 to 2.0 × 10^8 cpm/µg) as previously described (51). When the mutants were being examined for their ability to generate single-stranded DNA, the extraction procedure was modified as described by Carter et al. (16) to minimize DNA reannealing. Plasmid DNA was isolated as described previously (58).

Construction of mutants. Form I pSM620 plasmid DNA was partially digested with HaeIII, BstNI, PstI, NcoI, or ApaI, and completely digested with XhoI. Table 1 indicates the restriction enzyme(s) used for each mutant. The products of the digestions were fractionated on 1.0% agarose gels, and full-length or nearly full-length linear DNA was extracted by electroelution (51) and concentrated by passage through Elutip-d columns (Schleicher & Schuell, Inc.). In the case of the XhoI and ApaI fragments, the linear DNA was ligated to circles and cloned (see below) without further modification. In the case of the remaining fragments, one of two foreign DNA fragments was inserted before circularization and cloning. The foreign DNA inserts were either an 8-base pair (bp) oligonucleotide linker that contained the BglII site or a 263-bp CfoI fragment that contained the gene for a suppressor tyrosine tRNA. The latter fragment was isolated from the plasmid pSV-tT-2 (Su$^+$) constructed by Laski et al. (41) and kindly supplied by P. Sharp (Massachusetts Institute of Technology, Cambridge). If the termini of the fragment or insert contained either a 3' or 5' single-stranded DNA overhang, the ends were repaired with T4 DNA polymerase or the Klenow fragment in the presence of all four deoxynucleoside triphosphates (58) before ligation. Whenever the BglII linker was used, it was ^{32}P-labeled at its 5' ends with T4 polynucleotide kinase before ligation so that the blunt-end ligation could be monitored by autoradiography. After blunt-end ligation, the products of the reaction were digested to completion with BglII and precipitated with ethanol to ensure that only one linker was inserted in each mutant. The BglII linears were then religated back to circles before cloning. Finally, the products of each ligation were transfected into E. coli HB101 as described previously (58), and the individual clones were examined for the presence of the desired mutant plasmid by restriction enzyme analysis.

Mutants that contained a net gain of nucleotides were designated insertion mutants (ins) and named by the map position of the insertion (Table 1). Mutants that suffered a net loss of nucleotides were designated deletion mutants (dl)

TABLE 1. Physical structure of AAV mutants

Mutant[a]	Restriction site[b]	Bp deleted[c]	Bp inserted[c]	Nucleotide no.[d]
ins11 (pHM1412)	PstI	4	8	499
ins13 (pWM108)	NcoI	0	263	625
ins23 (pHM1505)	BstNI	0	9	1059
ins27 (pHM322)	HaeIII	0	8	1278
ins32 (pHM326)	HaeIII	0	8	1488
ins42 (pHM1411)	PstI	4	8	1962
ins49 (pHM1523)	BstNI	0	9	2282
ins52 (pHM347)	HaeIII	0	8	2416
ins55 (pHM1551)	BstNI	0	9	2561
ins63 (pHM805)	HaeIII	0	8	2945
ins78 (pHM1508)	BstNI	0	9	3639
ins86 (pHM1536)	BstNI	0	9	4002
ins80tsup (pWM106)	NcoI	0	263	3762
ins91 (pHM1410)	PstI	4	8	4258
dl3-23 (pHM1515)	BstNI	915	9	144–1059
dl10-37 (pHM334)	HaeIII	1,251	8	486–1737
dl23-28 (pHM324)	HaeIII	236	8	1086–1322
dl48-52 (pLB202)	XhoI	186	0	2224–2419
dl49-55 (pHM1549)	BstNI	279	9	2282–2561
dl49-94 (pHM3305)	BstNI, HinfI	2,108	8	2282–4391
dl52-91 (pHM1320)	HaeIII, PstI	1,842	8	2416–4258
dl58-87 (pHM401)	HaeIII	1,351	8	2691–4042
dl63-86 (pLB101)	ApaI	1,097	0	2947–4044
dl80-86 (pLB102)	ApaI	281	0	3763–4044
dl80-96 (pLB314)	NcoI	722	0	3762–4484

[a] Each mutant was designated as an insertion (ins) or deletion (dl) at a particular map position. Numbers in parentheses are laboratory isolation numbers, which are included in the mutant name to prevent possible confusion between two mutants isolated at the same position.

[b] Restriction site indicates the initial restriction enzyme used in the construction of the mutant.

[c] Bp deleted or inserted were estimates based on the restriction enzyme analysis of the mutant and the mode of construction. For example, dl23-28 (pHM324) was apparently the result of the deletion of the HaeIII fragment containing nucleotides 1086 to 1322, followed by the insertion of the 8-bp BglII linker fragment.

[d] Nucleotide numbers are those of Srivastava et al. (66), except for mutants dl80-86. The ApaI site at map position 80 was not present in the DNA sequence, and its position was estimated by restriction enzyme analysis (M. A. Labow and K. I. Berns, unpublished data). The numbers given for each mutant indicate the nucleotide numbers of the restriction sites used for the construction of each mutant.

and named by the range of the deletion in map units. In all cases, the laboratory isolation number was included in the name (see numbers in parentheses, Table 1) to avoid possible confusion between two different mutants with mutations at the same map position.

Although none of the mutants were sequenced, it was possible to deduce the probable nature of the mutation on the basis of the known properties of the enzymes used in the construction of each mutant (Table 1) and the known DNA sequences of AAV (66) and the inserts (41). For example, in the case of ins11 (pHM1412), the wild-type circular plasmid, pSM620, was opened at AAV nucleotide 495 and the PstI ends were treated with T4 DNA polymerase to remove the 4-bp single-stranded 3' overhang left by PstI. The addition of the 8-bp BglII linker resulted in the net insertion of 4 bp at AAV map position 11 (i.e., a frameshift mutation). In the case of ins23, the repair of the 1-bp 5' overhang left by BstNI and the addition of the 8-bp BglII linker led to a net (inphase) insertion of 9 bp at map position 23. The information for the remaining mutants is tabulated in Table 1.

Finally, some of the mutants reported here were the result of reconstruction from preexisting mutants. These were as follows: dl52-91 (pHM1320) was constructed by digesting ins52 and dl0-91 with BglII and EcoRV and ligating the

resulting fragments; *dl*49-94 (pHM3305) was constructed by adding *Bgl*II linkers to the 1,700-bp *Hinf*I fragment of pSM620 (containing AAV m.u. 94 to 100 and adjacent pBR322 sequences) and digesting the products with *Eco*RV and *Bgl*II. This restriction fragment mixture was then ligated to a *Bgl*II-*Eco*RV double digest of *ins*49.

RESULTS

Construction and physical characterization of the mutants. Insertion mutants were constructed by a procedure first described by Heffron et al. (29). The wild-type AAV plasmid, pSM620, was converted to linear DNA by partial digestion with any one of several multiple-cut restriction enzymes (Table 1). Whenever necessary, single-stranded ends were converted to duplex DNA by treatment with T4 DNA polymerase (or the Klenow fragment), and an 8-bp *Bgl*II linker fragment was then inserted at the position of the mutation. Most of the mutants made by this procedure were expected to be frameshift mutants, but a few (specifically, those in which *Bst*NI was used to cut pSM620) were expected to produce in-phase 9-bp insertions (Table 1). Finally, many mutants were isolated that contained a deletion between two restriction sites within the AAV genome as well as a *Bgl*II linker insertion. The *Bgl*II linker was chosen for these experiments because the site was not present in the wild-type plasmid. This both facilitated the restriction enzyme mapping of the mutations (data not shown) and provided a single-cut restriction site for the insertion of foreign DNA (P. L. Hermonat and N. Muzyczka, unpublished data). In addition to the mutants containing *Bgl*II linkers, two other classes of mutants were made. The first had simple deletions between existing restriction sites within the genome, such as *dl*48-52. The second class involved the insertion of a 263-bp DNA fragment that contained a tyrosine suppressor tRNA gene constructed by Laski et al. (41). Insertion mutant *ins*80tsup is an example of this class.

The details of the construction of each mutant are summarized in Table 1. It should be pointed out that none of the mutations were sequenced and, therefore, the nucleotide numbers and base pairs cited in Table 1 are estimates based on the restriction enzyme analysis of the mutants (data not shown) and on the manner in which each mutant was constructed (see above). Figure 1 shows the positions of the mutations, which will be discussed in detail.

Mutants defective for duplex DNA replication. Initially, the mutants were tested for their ability to rescue and replicate the AAV sequences when the plasmid DNA was transfected into human cells by using the DEAE-dextran procedure (49). Human Ad5-transformed 293 cells or HeLa cells were used as the host, and Ad2 was used as the co-infecting helper virus. Typically, Hirt supernatant DNA (30) was harvested at 36 to 48 h postinfection and analyzed by Southern blotting, using ^{32}P-labeled AAV DNA as probe. The results are shown in Fig. 2 for a representative selection of the mutants, and a summary of the results for all of the mutants is listed in Table 2.

Approximately half of the AAV-2 genome appeared to be involved in the rescue or replication of AAV DNA. Mutants with mutations between map positions 11 and 42 were defective for the production of monomer duplex AAV DNA after plasmid transfection (*rep* mutants). In most cases, only input plasmid AAV sequences could be detected, and no evidence of DNA replication was seen. The two exceptions were *ins*23 and *ins*42, which were capable of producing yields of monomer duplex AAV DNA that were 0.1 to 1.0% of that seen in wild-type-infected cells (Fig. 3). One of these
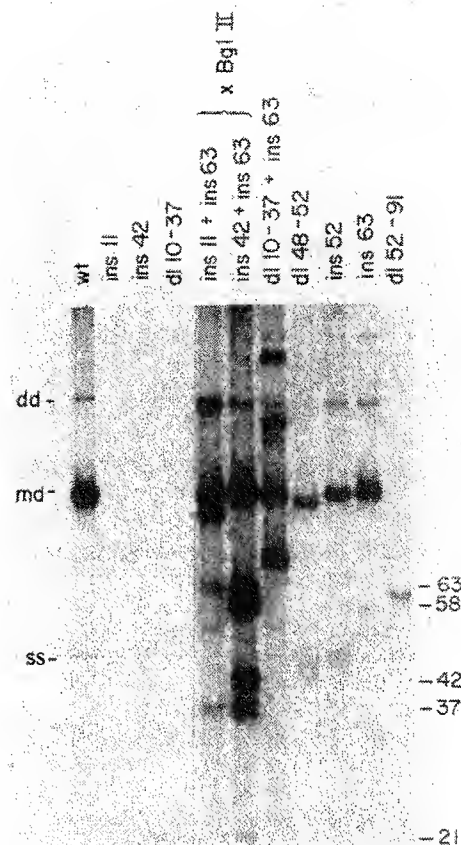

FIG. 2. DNA replication phenotypes of AAV mutants. One microgram of form I supercoiled plasmid DNA was transfected into 293 cells in single plasmid assays. When two plasmids were transfected together, 10 μg of the replication-defective mutants (*ins*11, *ins*42, *dl*10-37) was mixed with 1 μg of *ins*63 before transfection. The cells were subsequently infected with Ad2 helper virus at a multiplicity of infection of 5. At 36 to 48 h, cells were lysed and treated with pronase, and low-molecular-weight DNA was isolated by Hirt extraction (30). One-tenth of each DNA extract was then fractionated on a 1.4% agarose gel, transferred to nitrocellulose by the method of Southern (65), and hybridized to nick-translated, ^{32}P-labeled AAV DNA. Where indicated, the extracts were digested with *Bgl*II before electrophoresis. *dl*52-91 appeared to replicate at a lower level in this experiment, but other experiments (data not shown) indicated that its replication was similar to that of other *lip* and *cap* mutants. *ins*42 appeared not to replicate in this experiment but was subsequently found to replicate at very low levels (see Fig. 3). The positions of the dimer duplex replicative form (dd), monomer duplex replicative form (md), and single-stranded form (ss) are indicated. Also, the sizes of the fragments in the *ins*42 and *ins*63 experiment (lane 6) are indicated as a percentage of the full-length AAV size.

mutants (*ins*23) also produced detectable levels (>0.01% of wild type) of infectious virions (Table 2).

In general, mutants with mutations on the right side of the AAV genome (map positions 48 to 93) were capable of generating normal replicating pools of AAV DNA (i.e., monomer and dimer duplex DNA). As expected, deletion mutants with mutations in this region generated DNA molecules that were shorter than wild-type AAV DNA by the appropriate amount (*dl*48-52 and 52-91 in Fig. 2). The only mutant with a mutation on the right side of the genome found to be defective for replication (5% of wild type) was *dl*80-96 (Table 2). Although this mutant was missing a portion of the

TABLE 2. Phenotypes of AAV-2 mutants

Mutant	Duplex DNA replicators[a]	Single-stranded progeny[a]	Virus yield (%)[b]	Pheno-type
*ins*11 (pHM1412)	−	−	ND[c]	Rep
*ins*13tsup (pWM108)	−	−	ND	Rep
*ins*23 (pHM1505)	± (0.1–1.0)	+	0.1	Rep
*ins*27 (pHM322)	−	−	ND	Rep
*ins*32 (pHM326)	−	−	ND	Rep
*ins*42 (pHM1411)	± (0.1)	ND	0[d]	Rep
*ins*49 (pHM1523)	+	ND	0.1–1.0	Lip
*ins*52 (pHM347)	+	+	0.1–1.0	Lip
*ins*55 (pHM1551)	+	+	0.1–1.0	Lip
*ins*63 (pHM805)	+	−	0	Cap
*ins*78 (pHM1508)	+	−	ND	Cap
*ins*80tsup (pWM106)	+	−	ND	Cap
*ins*86 (pHM1536)	+	−	ND	Cap
*ins*91 (pHM1410)	+	ND	0	Cap
*dl*3-23 (pHM1515)	−	−	ND	Rep
*dl*10-37 (pHM334)	−	−	0	Rep
*dl*23-28 (pHM324)	−	−	ND	Rep
*dl*48-52 (pLB202)	+	+	0.1–1.0	Lip
*dl*49-55 (pHM1549)	+	ND	0.1–1.0	Lip
*dl*49-94 (pHM3305)	+	−	0	Cap
*dl*52-91 (pHM1320)	+	−	0	Cap
*dl*58-87 (pHM401)	+	−	ND	Cap
*dl*63-86 (pLB101)	+	−	ND	Cap
*dl*80-86 (pLB102)	+	−	ND	Cap
*dl*80-96 (pLB314)	±(5)	ND	ND	Rep

[a] Duplex or single-stranded DNA replication was judged by the presence of monomer single-stranded or duplex AAV DNA in Hirt supernatant DNA after transfection into 293 or HeLa cells with Ad2 as the helper virus. (See the legends to Fig. 2, 3, and 4 and the text). Numbers in parentheses indicate the yield of monomer duplex AAV DNA as a percentage of wild-type levels (see Fig. 3).

[b] Virus yield refers to the yield of infectious virus from cells transfected with mutant plasmids and harvested at 48 h postinfection. (See the text for additional details.) The yield is expressed as a percentage of the wild-type plasmid (pSM620) yield.

[c] ND, Not determined; in the case of single-stranded progeny DNA, the DNA isolation was not done under conditions that inhibited reannealing.

[d] The limit of detection was a virus yield 0.01% that of wild type.

AAV capsid gene VP3 (see below), its replication defect probably was due to the fact that *dl*80-96 was missing the polyadenylation signal (95 m.u.) that is used in all known AAV RNAs.

Mutants defective in generating single-stranded progeny DNA. Because AAV virions contain single-stranded DNA, we tested the DNA-positive mutants for their ability to generate single-stranded progeny DNA. Hirt supernatant DNA was isolated from cells transfected with mutant plasmids under conditions that minimized AAV DNA re-annealing (16), and then was analyzed by Southern blotting (65). All of the replication-positive mutants with mutations between map positions 63 and 91 were defective in generating single-stranded progeny DNA (Table 2). Single-stranded AAV DNA was identified by its mobility during electrophoresis compared with virion DNA (data not shown) and by its resistance to digestion with *Pst*I. Figure 4 shows the results that were obtained when *ins*80tsup was transfected into human cells. Although the mutant was capable of synthesizing monomer duplex DNA to approximately the same level as wild type, it was clearly deficient in generating single-stranded AAV DNA. Similar results are shown for *ins*63 in Fig. 2. Thus, the phenotype of a mutant with a mutation in the major AAV capsid protein, VP3, appeared to be an inability to synthesize or maintain single-stranded DNA. As might be expected, mutants of this class produced no

detectable infectious virions (Table 2). Because these mutants all had mutations that mapped within the open reading frame that is believed to code for the most abundant AAV capsid protein, VP3, we called these mutants capsid (*cap*) mutants.

Mutants that synthesized double- and single-stranded AAV DNA but produced low virus yields. AAV mutants with mutations between map positions 48 and 55 were positive for the production of both duplex and single-stranded AAV DNA but produced only 0.01 to 1.0% of the normal virus yield (Table 2 and Fig. 5). The virus yield for this class of mutants was estimated by collecting the virus from cells that had been transfected with equivalent amounts of wild-type or mutant plasmid DNA. The virus stocks were then titrated by infecting human cells and comparing the yields of monomer duplex DNA produced by mutant and wild-type AAV stocks (Fig. 5). Alternatively, the stocks were titrated by counting the number of cells that became positive by indirect immunofluorescence for AAV capsid antigens after infection (data not shown). The two procedures gave comparable virus titers. To distinguish these mutants from *cap* mutants, we called them low infectious particle yield (*lip*) mutants.

Complementation and recombination between mutant phenotypes. To determine whether the replication-deficient (*rep*) mutants could be complemented by mutants that were positive for DNA replication, representatives of the two classes of mutants were mixed and the plasmid DNA mixture was transfected into human cells. Low-molecular-weight DNA was then harvested and examined by agarose gel electrophoresis to determine whether mutant genomes were capable of rescue and replication. In those cases in which both mutants were *Bgl*II linker insertion mutants, the DNA extracts were digested with *Bgl*II before electrophoresis to determine the extent of replication of each mutant (Fig. 2). In all cases in which a DNA-positive mutant was used to complement a *rep* mutant, replication of both genomes was
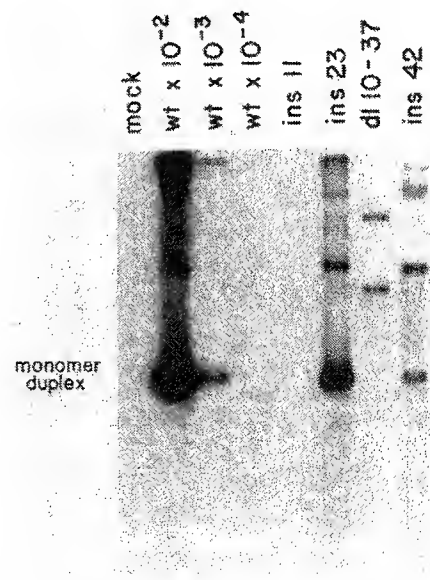

FIG. 3. Mutants partially defective for DNA replication. Procedures were the same as those for Fig. 2. Ten-fold dilutions of the wild-type (pSM620) plasmid extract were electrophoresed to permit quantitation of the level of rescue and DNA replication of mutants *ins*23 and *ins*42. The two bands observed in *dl*10-37 transfections represented form II (nicked circular) and form III (linear) input plasmid DNA.
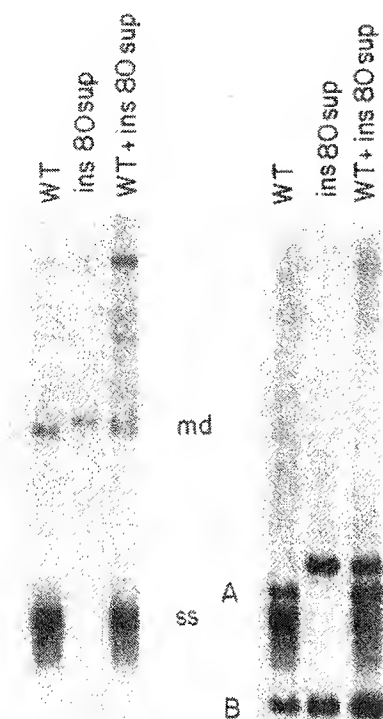
FIG. 4. Production of single-stranded AAV DNA by wild-type and *cap* mutants. Human 293 cells were transfected with 5 μg of the wild-type plasmid (pSM620), 5 μg of the *cap* mutant *ins*80tsup plasmid, or both, as described in the text. At 40 h postinfection, low-molecular-weight DNA was extracted by a procedure that minimized re-annealing of single-stranded DNA (16). Samples of the DNA extracts were fractionated on 1.4% agarose gels (left panel) or digested with *Pst*I before electrophoresis (right panel). After electrophoresis, the DNA was transferred to nitrocellulose and hybridized with nick-translated, ^{32}P-labeled AAV DNA. Because *ins*80tsup is 263 bp larger than wild-type AAV, the monomer duplex DNA produced by this mutant had a slower mobility during electrophoresis (left panel). Similarly, because the insertion was located in the AAV *Pst*I A fragment, this fragment is missing in *ins*80 *Pst*I digests and is replaced by a slightly larger fragment (right panel). The mobilities of monomer duplex (md) and single-stranded (ss) AAV DNA, as well as of the wild-type *Pst*I A and B restriction fragments, are indicated.

observed (Fig. 2 and Table 3). For example, in the *ins*42–*ins*63 complementation (Fig. 2), five major *Bgl*II restriction fragments were observed. These were: an apparently uncut full-length AAV DNA fragment; a 63% (of full AAV size) fragment and a 37% fragment that were characteristic of the *ins*63 mutant; and the 42 and 58% fragments that were characteristic of *ins*42. The large amount of apparently full-length AAV DNA that was not cut by *Bgl*II was probably the result of single-stranded *ins*42 DNA and *ins*63 DNA rean-nealing to form a duplex DNA species. This duplex DNA would be resistant to digestion at both potential *Bgl*II sites.

It is worth noting that in addition to the expected *Bgl*II fragments, a faint 21% fragment was consistently observed in *ins*42–*ins*63 complementations. This was the appropriate size for a *Bgl*II fragment that would be present in the double mutant (*ins*42 + *ins*63) recombinant. The yield of this fragment, compared with that of the parental *Bgl*II fragments

(approximately 1%), could be interpreted as an estimate of the recombination frequency during one cycle of lytic growth. Similar double mutant recombinant bands (of the appropriate size) were seen when complementation experiments were performed with *ins*63 and *ins*11 (Fig. 2), as well with as other pairs of mutants (data not shown).

As described above, two types of mutants were found which appeared completely viable for duplex DNA replication, i.e., *lip* and *cap* mutants. Both types of DNA-positive mutants were capable of complementing *rep* mutants (Table 3).

Conversely, most *rep* mutants could complement the defect in *cap* mutants (Table 3). An example of this is shown in Fig. 6 for the capsid mutant *ins*63. When it was transfected by itself, *ins*63 produced no detectable AAV virus, but complementation with any one of three *rep* mutants (*ins*11, *dl*10-37, or *ins*42) produced mixed virus stocks with variable amounts of *ins*63 mutant DNA (Fig. 6).

Because some of the *rep* mutants retained limited ability to rescue and replicate AAV DNA, a number of complementation experiments were performed to determine whether there might be two or more *rep* complementation groups. None of the *rep* mutants could complement other mutants of the same phenotype, regardless of whether the defect in replication was complete or partial (Table 3).

An attempt was also made to distinguish two complementation groups within the mutants that were DNA positive but produced little or no infectious virus (namely, *lip* and *cap*
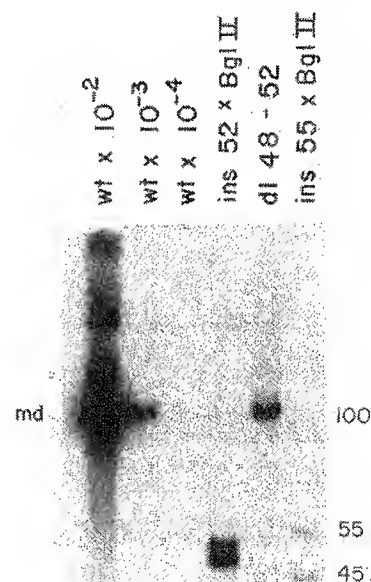

FIG. 5. Infectious virus production by *lip* mutants. Human D6 cells that had been transfected with mutant plasmids or the wild-type plasmid (pSM620) were harvested at 48 h posttransfection, freeze-thawed three times, and heated to 56°C for 30 min. One-third of the virus stocks were then used to infect 10-cm dishes of D6 cells. At 36 h postinfection, DNA was harvested from the infected dishes and analyzed as in Fig. 2. Mutant lanes represent 10% of the DNA recovered from one 10-cm dish (lanes 4 to 6). Lanes 1 to 3 represent the indicated dilutions of 10% of the DNA recovered in the wild-type extract. Numbers at right indicate the positions of the full-length monomer duplex (100%) and the *Bgl*II restriction fragments in the *ins*55 lane as a percentage of the full-length genome.

TABLE 3. Complementation[a] of AAV mutants

Complementing genome	Complementation with:													
	ins11 (rep)	ins13 (rep)	ins23 (rep)	ins27 (rep)	dl23-38 (rep)	dl10-37 (rep)	ins32 (rep)	ins42 (rep)	ins52 (lip)	ins63 (cap)	ins78 (cap)	dl80-96 (rep)	ins86 (cap)	ins91 (cap)
Wild type		+			+	+	+					+		
ins11 (rep)					−		−	−		+				
ins23 (rep)	−							−		+				
ins27 (rep)	−							−						
dl10-37 (rep)										+				
ins32 (rep)	−	−						−						
ins42 (rep)	−	−	−					−		+				
ins52 (lip)				+		+				−				
ins55 (lip)						+								
ins63 (cap)	+		+	+	+	+	+	+	−					−
ins78 (cap)	+					+		+						−
dl80-96 (rep)						−								
ins86 (cap)														−
ins91 (cap)										−	−		−	

[a] The table indicates whether wild-type or a mutant plasmid DNA, indicated in the left column, could complement the defect in the mutants listed at the top of the table. When the partially viable *rep* mutants *ins*23, *ins*42, and *dl*80-96 were used to complement other *rep* mutants, both genomes replicated at the level of the partially viable mutant. However, no evidence was seen for either intergenic or intragenic complementation.

mutants). An attempt at complementing mutants of these two classes with each other was unsuccessful (Table 3). Thus, although it was possible to distinguish two mutant phenotypes among the mutants with mutations between map positions 48 and 91, we could not demonstrate two distinct complementation groups. We concluded that the *lip* function must share a common coding region with *cap* and that both *lip* and *cap* functions were required to produce a normal yield of infectious virus particles.

DISCUSSION

We isolated deletion and insertion mutants which had mutations in most of the major open reading frames in the AAV genome. Among these mutants, we distinguished at least three phenotypes: (i) mutants that were defective in their ability to rescue or replicate the AAV genome from the recombinant plasmids (*rep* mutants); (ii) mutants that synthesized normal amounts of AAV duplex DNA but did not generate single-stranded virion DNA (*cap* mutants); and (iii) mutants that synthesized normal amounts of AAV single-stranded and duplex DNA but produced substantially lower yields of infectious virus particles than did wild-type AAV (*lip* mutants).

rep **mutants.** Because AAV relies heavily on helper and host functions during its lytic cycle, it has been an open question whether AAV contains any genes required for its own DNA replication. The fact that some kinds of defective interfering particles were incapable of DNA replication unless wild-type AAV was also present led to the suggestion that AAV codes for a *rep* function (42). Because the defective interfering virus stocks used were composed of a heterogeneous mixture of AAV deletions, it was not clear which part of the genome might be responsible for DNA replication. The first indication that a particular region of the AAV genome may be necessary for DNA replication came from DNA transfection experiments in which a mutant with an in vitro-constructed deletion (38 to 43 m.u.) was found to be incapable of DNA replication unless wild-type AAV DNA was cotransfected (4; Cheung, Ph.D. dissertation). In retrospect, this particular deletion was likely to have reduced the expression of several AAV gene products because it removed one of the AAV splice junctions. More recently, when Srivastava et al. (66) completed the AAV DNA sequence and discovered a large open reading frame on the
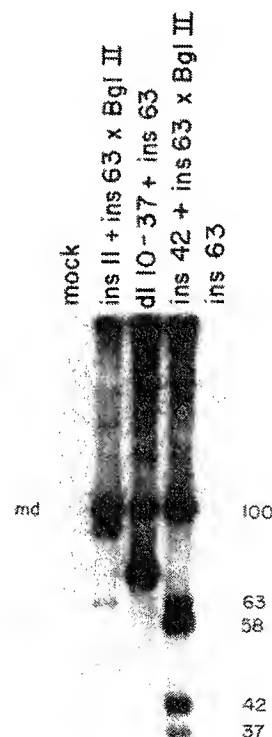
FIG. 6. Complementation of capsid mutants by *rep* mutants. Virus production was assayed by repeating the complementation experiment as in Fig. 2, with the following changes. At 48 h postinfection, cells were freeze-thawed three times and heated to 56°C for 30 min. One-third of the virus stock was used to infect a single 10-cm plate of 293 cells, and the cells were then infected with Ad2 at a multiplicity of infection of 5. At 36 h postinfection, DNA was harvested and analyzed as in Fig. 2. Each lane represents 10% of the DNA recovered from one 10-cm dish. Wherever indicated, the DNA extracts were digested with *Bgl*II before electrophoresis. The sizes of the fragments in the *ins*42 plus *ins*63 lane are indicated as percentages of the full-length AAV duplex DNA.

left side of the genome, it was suggested that this open reading frame is involved in AAV DNA replication. Our mutant studies indicate that AAV does, indeed, have at least one gene that codes for a DNA replication function; its location is in the left half of the AAV genome between map positions 11 and 42.

Although we have demonstrated unequivocally that AAV has genes that are involved in its own replication, it is still not clear how many rep genes there are. The fact that two spliced RNAs are synthesized from the left half of the genome (45) suggests that at least two proteins are coded by the spliced open reading frames that are present in the 3.3- and 3.9-kb RNAs (Fig. 1). However, because the reading frames in these two RNAs were overlapping and in phase with each other, it was not possible by our mutagenesis procedure to target a mutation that would be exclusively within the 3.3-kb RNA. All of the mutants with mutations between 21 and 48 m.u. were expected to inactivate both hypothetical proteins. For the same reason, the fact that complementation experiments revealed only one rep complementation group does not rule out the possibility that there is more than one rep gene. Finally, a peculiarity of our DNA replication assay should be mentioned. For a mutant to be scored as DNA positive, two events must have occurred after plasmid DNA transfection: the mutant had to be rescued so that it was free of pBR322 sequences, and then it had to undergo DNA replication. If the larger hypothetical protein were involved specifically in the rescue of AAV sequences, then it would be difficult to distinguish any other

phenotypes among the mutants with mutations on the left side of the genome.

All of the points made above about the spliced 3.3- and 3.9-kb RNAs can be made, as well, about the hypothetical proteins coded within the unspliced 2.6-, 3.6-, and 4.2-kb RNAs (Fig. 1). The question of whether the unspliced transcripts are functionally active is raised by the phenotypes of the partially viable rep mutant, ins42, and the rep⁺ mutant dl48-52. ins42 had a mutation within the major AAV intron (41 to 48 m.u.) and was, therefore, expected to be a viable mutant. The fact that DNA replication is severely depressed by the ins42 mutation indicates either that one of the coding regions present in the unspliced RNAs is functional or that the mutation has an unforeseen effect on RNA splicing.

In this regard, it is worth mentioning the phenotype of the lip mutant dl48-52. This mutant is missing the last four C-terminal amino acids of the coding potential in the spliced 3.3- and 3.9-kb RNAs. These have been replaced with 71 amino acids that are not normally present in these coding regions. Despite this, the DNA replication phenotype of dl48-52 appears to be normal. This suggests also that the spliced 3.3- and 3.9-kb RNAs are not the AAV mRNAs required for DNA replication. An alternative explanation for the dl48-52 phenotype is that the C-terminal end of these proteins is dispensable for DNA replication.

cap mutants. The mutation responsible for the cap mutant phenotype maps within the coding region for the major AAV capsid protein, VP3 (35, 66). This protein represents 85% of
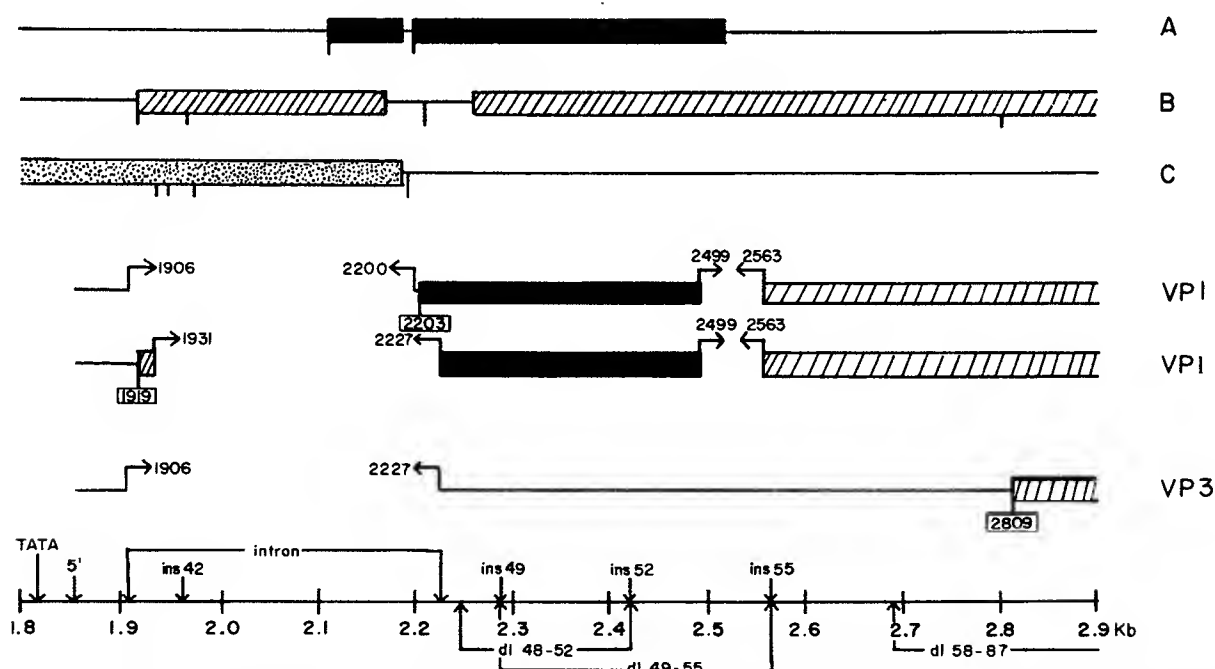

FIG. 7. Potential open reading frames in the lip region. The top three lines indicate the positions of the open reading frames in the A (filled boxes), B (striped boxes), and C (stippled box) reading frames between nucleotides 1800 and 2900. Vertical lines indicate the positions of potential ATG start codons in the three reading frames. The bottom line shows the positions of the mutations in this region as well as the positions of the 5' terminus, intron, and the TATA site for the known 2.3-kb mRNA. The VP3 line indicates the structure of the 2.3-kb mRNA that codes for VP3. The striped box indicates the VP3 coding sequences. The vertical line and boxed number indicate the position of the VP3 ATG start codon. Bent arrows and associated numbers indicate the nucleotide positions of the 2.3-kb splice junction. The VP1 lines indicate the structures of mRNAs that theoretically could code for VP1. None of these mRNAs have been observed in AAV-infected cells. Their existence is inferred from mutant and protein studies described in the text. The symbols used are the same as those described for the other lines above.

the total AAV capsid protein, and thus it is unlikely that AAV capsids are assembled by *cap* mutants. AAV is thought to replicate by a strand displacement mechanism from either terminus, so that both plus and minus strands are equally likely to be displaced (4, 27, 46, 47, 63, 64, 67). The fact that *cap* mutants synthesize normal levels of the monomer duplex replicative intermediate (Fig. 2) indicates that a preformed capsid probably is not directly required during strand-displacement synthesis. If capsids were, in fact, necessary for replication, it would be unlikely that monomer duplex DNA would accumulate in *cap* mutants. A more likely explanation for the inability of cap mutants to generate single-stranded DNA is that unless the displaced progeny strand is sequestered immediately within a capsid, it is recycled into the replicative intermediate DNA pool (53). In this respect, AAV *cap* mutants are reminiscent of the capsid mutant phenotypes of some procaryotic single-stranded DNA phages such as ϕX174 (28).

The phenotype of the *cap* mutants was anticipated during genetic studies of the helper functions supplied by adenovirus. Carter and his colleagues discovered that when the mutant Ad5*ts*125 was used in coinfections with AAV at a nonpermissive temperature, it was defective for AAV helper function (54). Ad5*ts*125 has a mutation in the adenovirus 72-kilodalton DNA binding protein that is required for adenovirus DNA replication (20, 68) and has pleiotropic effects on the expression of other adenovirus genes (17, 39, 55). When the *ts*125 defect for AAV helper function was examined further, it was discovered that duplex AAV DNA replication and transcription were essentially normal in *ts*125-infected cells but that the synthesis of AAV capsid proteins and the accumulation of single-stranded AAV DNA were both significantly reduced (13, 35, 53, 54). It was suggested, therefore, that unless AAV-infected cells synthesized the AAV capsid proteins, single-stranded AAV DNA would not accumulate (53). We have essentially confirmed and extended these observations by isolating mutants with mutations directly within the major AAV capsid gene.

Now that it is established that AAV codes for a protein required for its own DNA replication, an additional point can be made about the *ts*125 observations. Unlike the capsid proteins, translation of the AAV gene product(s) required for DNA replication apparently is not affected by the *ts*125 mutation. In contrast, all of the AAV mRNAs require the expression of adenovirus early region 1A or 1B or both for transcription (15, 33, 34, 56, 59, 60). Thus, although the AAV lytic cycle cannot be divided into conventional early and late phases, the expression of AAV gene products does appear to be differentially regulated.

Finally, it should be mentioned that the synthesis of VP3 or preformed capsids is probably not the only mechanism for controlling the ratio of single- to double-stranded AAV DNA in infected cells. If it were, then cells infected with *cap* mutants would accumulate much higher pools of monomer duplex DNA than are seen in wild-type infections. The fact that *cap* mutants synthesize nearly normal levels of duplex AAV DNA suggests that there is a more subtle regulation of replicating AAV DNA pools.

lip mutants. Several lines of evidence indicate that *lip* mutants are defective in the minor AAV capsid protein, VP1. First, the phenotypes of *lip* mutants suggest that they are the result of a defect in a virion structural protein or a capsid assembly protein. Second, the fact that *lip* mutants cannot complement *cap* mutants suggests that these mutants share some of their coding sequences and, therefore, that *lip* mutants are probably defective in one of the minor AAV

capsid proteins, VP1 or VP2. Finally, recent observations (McPherson and Rose, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, S25, p. 288; J. E. Janik, M. M. Huston, and J. A. Rose, personal communication) indicate that at least one *lip* mutant (*dl*48-52) is, in fact, a VP1 mutant. Janik et al. independently isolated an AAV mutant that contained a deletion identical to the one in *dl*48-52 as well as deletions within the AAV terminal repeats. Examination of the capsid proteins synthesized in cells that were transfected with this mutant indicated that the mutation in *dl*48-52 produced an inphase deletion in VP1 but did not affect the molecular weights of VP2 and VP3. Thus, *lip* mutants appear to be VP1 mutants.

The significance of the *lip* phenotype, and of the observation of Janik et al., is that there must be additional AAV mRNAs that have not yet been observed. The known 2.3- and 2.6-kb mRNAs do not have open reading frames sufficiently large to code for VP1 (66). Because of the position of the *lip* mutations, the most likely explanation is that VP1 is translated from a transcript with introns different from the one that has been mapped in the 2.3-kb mRNA. This would allow the use of the coding region between 47 and 54 m.u. in which most of the *lip* mutations map.

To see whether we could predict the structure of the VP1 mRNA from the positions of the *lip* mutations, we examined the AAV DNA sequence between 1,800 and 2,900 bp (Fig. 7). Although there are more than 100 potential splice junctions in this region, there are relatively few ATG and nonsense codons that need to be considered. Indeed, only two mRNAs appeared to be reasonable based on the following additional criteria (Fig. 7). (i) All *lip* mutations must map within the VP1 coding region but outside VP3 coding sequences. (ii) The *rep* mutant *ins*42 maps outside the coding regions of all three AAV capsid proteins. (iii) A minimum of introns are used for the VP1 mRNAs. (iv) The theoretical molecular weights of the proteins coded by the VP1 mRNA must be as high as possible. The hypothetical VP1 mRNAs (Fig. 7) we have proposed are approximately 2.3 kb in length and code for proteins of 85 and 86 kilodaltons. These values agree reasonably well with the observed molecular weights of 85,000 to 90,000 for VP1 (9, 10, 35, 50, 62). Needless to say, other explanations for mutation responsible for the *lip* phenotype are possible. (For a brief discussion, see reference 66.)

Finally, although the precise function of VP1 is not known, the phenotype of the *lip* mutants is consistent with the results of studies on the assembly of AAV virions. Myers and Carter (52) found three types of AAV capsids during lytic infections as judged by their sedimentation in sucrose gradients. These were the 66S empty capsids, a 60S capsid intermediate that was associated with single-stranded DNA, and the 110S mature virions. Because *lip* mutants can accumulate single-stranded AAV DNA, VP1 is probably not required in the assembly of empty capsids or the 60S intermediates. It is possible, therefore, that VP1 plays a role in the conversion of the 60S intermediate to the mature virion.

In conclusion, we should mention that although we have identified at least three AAV genes, our collection of mutants is not exhaustive. Based on a variety of considerations (discussed above), we estimate we have obtained mutants with mutations in approximately half of the AAV genes. Certainly the complexity of the AAV life cycle (integration, rescue, replication, virion assembly, and helper and host interactions) suggests that additional virus-coded phenotypes remain to be discovered. Thus, although AAV was

once thought to be one of the simplest of all mammalian viruses, its genetic structure appears to be as complex as that of other viruses of comparable size (that is, papovaviruses and retroviruses).

ACKNOWLEDGMENTS

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